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# Abscisic Acid Levels and Seed Dormancy

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*Abstract.* **Dormant seeds from *Fraxinus* species require cold-temperature after-ripening prior to germination. Earlier, we found that abscisic acid (ABA) will inhibit germination of excised nondormant embryos and that this can be reversed with a combination of gibberellic acid and kinetin. Using Milborrow's quantitative "racemate dilution" method the ABA concentration in 3 types of *Fraxinus* seed and pericarp were determined. While ABA was present in all tissues, the highest concentration was found in the seed and pericarp of dormant *F. americana*. During the chilling treatment of *F. americana* the ABA levels decreased 37 % in the pericarp and 68 % in the seed. The ABA concentration of the seed of the nondormant species, *F. ornus*, is as low as that found in *F. americana* seeds after cold treatment. Experiments with exogenously added ABA solutions indicate that it is unlikely that the ABA in the pericarp functions in the regulation of seed dormancy. However, the ABA in the seed does seem to have a regulatory role in germination.**

Abscisic acid (ABA) (1) has been implicated in the regulation of leaf abscission, senescence, growth inhibition, and bud and seed dormancy. Up to now most of the studies have dealt with effects of exogenously added ABA. The few isolation studies that have been reported do not permit a quantitative correlation between endogenous ABA levels and a change in the physiological state of the test plant.

We are studying physiological and biochemical aspects of seed germination with dormant and nondormant *Fraxinus* (ash) seeds. Villiers and Wareing (11) have shown that the germination behavior of excised embryos from this genus depends on their previous history. Embryos from cold-temperature after-ripened seeds will germinate rapidly when placed in moist chambers at room temperature, while those from untreated seeds show only very limited germination during a 10 day period. In *Fraxinus excelsior* L., germination of dormant embryos can be induced either by leaching or with gibberellic acid, $GA_3$ (11). We found that we can prevent germination in nondormant *Fraxinus americana* L. and *Fraxinus ornus* L. embryos with exogenously added (RS)-ABA. Furthermore the ABA-induced inhibition is partially reversed by combinations of $GA_3$ and kinetin (9).

It is generally accepted that results obtained exclusively with exogenously applied growth substances are insufficient to establish a regulatory role for these substances in the intact organism. A minimum requirement for such a role would be to show the presence of the hormones in the intact plant and to demonstrate a correlation between hormonal concentration levels and physiological states. With the elegant "racemate dilution" method developed by Milborrow (7) it is now possible to obtain quantitative values for the ABA concentration in plants. With this procedure we have determined the (S)-ABA levels in pericarp and seed before and after cold-temperature after-ripening in *F. americana* and in nondormant *F. ornus*. Attempts were also made to evaluate the possible physiological significance of the changes in the ABA levels to dormancy regulation.

## Experimental

*Fraxinus americana* seeds grown in the United States during the 1965 season and *F. ornus* seeds, 1963-64 crop imported from Italy were purchased from F. W. Schumacher, Sandwich, Massachusetts. Excised embryos from *F. ornus* seeds germinated well even without cold-temperature after-ripening. For cold-temperature after-ripening, *F. americana* samaras (seeds with pericarps) were dusted with Difolatan 80 wettable, Chevron Chemical Company, Ortho Division, rinsed thoroughly after 24 hours with sterile tap water and stored 3 months at 5° with moist vermiculite in polyethylene bags. (RS)-ABA was prepared by the procedure of Cornforth *et al.* (4).

*Germination of Embryos.* Seeds were depericarped by hand and placed into tapwater for 24 hours at room temperature. The embryos were excised with a razor blade and placed on Whatman No. 1 filter paper in 5 cm petri dishes. Ten embryos per dish and 1 ml solution consisting of test substances dissolved in 0.01 M potassium phosphate, pH 6.0, were used. Triplicate plates were held at 22° in a laboratory where the light came through large windows that faced north. The formation of a curvature at the basal end was interpreted as a

positive germination response. Following the recommendation of Timson (10) we are reporting the germination data as the sum of the daily percentage of germination for a 10 day period starting at the day of excision. Thus, $\Sigma 10 = 1000$ would mean 100 % germination during the first day. Chlorophyll content was determined spectrophotometrically from acetone extracts (6).

*Quantitative Determination of (S)-Abscisic Acid.* With only minor modifications we used the "racemate-dilution" method as described by Milborrow (7). This procedure rests on the fact that the enantiomorphs of ABA have extremely high optical rotation at the 2 extrema in the UV region; the specific rotation is 69000°. A known amount of (RS)-ABA is added to the plant material to be analyzed and the ABA purified until a characteristic ultraviolet curve can be observed. From this measurement one obtains the total ABA recovered while the optical rotatory dispersion (ORD) curve yields the amount of (S)-ABA isolated. The amount of (S)-ABA in the original sample can be calculated from the ratio of these 2 measurements and the known amount of (RS)-ABA added (7).

The (S)-ABA content of the following samples were determined: dry pericarp from *F. americana* and *F. ornus*, 24 hour-imbibed seed from *F. americana* and *F. ornus* that did not have any low temperature treatments, and pericarp and seed from *F. americana* that had been held at 5° for 3 months. Dry weights were obtained from aliquots of each sample by drying to constant weight at 110°.

To a weighed quantity of pericarp or seeds a known amount of (RS)-ABA was added during the extraction with 80 % methanol. The ether-soluble acids were separated as described by Milborrow (7). Significant purification could be obtained by use of a charcoal column adapted from the procedure of Ohkuma *et al.* (8), before thin layer chromatography. Carbon (Darco G-60) and acid-washed Celite (1:2 by weight) were mixed in water and packed into a 10 mm column. The column was eluted stepwise with 30 %, 40 %, 50 %, 60 %, and 100 % aqueous acetone. Each of the eluting fractions consisted of 30 ml solvent per gram of charcoal. Bioassays with wheat showed practically all of the growth inhibitory activity in the 50 % and 60 % acetone fractions. These were combined, the solvent separated at reduced pressure below 30°, and the residue applied to TLC plates using the systems described by Milborrow. Thin layer plates were washed 3 times in distilled ethanol and 2 times in distilled chloroform. Extracts from *F. americana* seed and pericarp were chromatographed twice; once in *n*-butanol-*n*-propanol-ammonia-water (2:6:1:2 v/v), a second time in benzene-ethyl acetate-acetic acid (50:5:2 v/v). *F. ornus* extracts from both pericarp and seed contained a larger amount of UV absorbing material, and a third chromatographic separation, again using the first solvent system, was necessary. After elution of the ABA with 95 % ethanol, the solvent was evaporated under reduced pressure, the residue taken up in dilute acid and the ABA extracted into ether. After evaporation of the ether, the residue was dissolved in a known volume of 0.005 M sulfuric acid in 95 % ethanol and the UV and ORD spectra obtained.

## Results

During cold-temperature after-ripening the ability of excised embryos of *F. americana* seeds to germinate increases drastically as indicated by a rise of $\Sigma 10$ from 0 to 30 to 700 to 950, table I. Embryos from *F. ornus* while not requiring prechilling of the seeds do not germinate during the first 2 or 3 days after excision and therefore their $\Sigma 10$ is usually not above 750. However, in the presence of 10 $\mu$M

Table I. *(S)-Abscisic Acid Content of Seeds and Pericarps of* Fraxinus americana *and* Fraxinus ornus

| Biological material | Dry wt | Germination as $\Sigma$ 10 | (RS)-ABA added | Total ABA recovered[3] | (S)-ABA recovered[4] | Total (S)-ABA present in sample[1] | | |
|---|---|---|---|---|---|---|---|---|
| | % | | | $\mu g$ | $\mu g$ | $\mu g/kg$ | $\mu moles/kg$ | $\mu g/1000$ seeds or pericarps |
| *F. americana* | | | | | | | | |
| Dormant seed | 98 | 0-30 | 50 $\mu g/200$ g[2] | 44 | 28 | 450 | 1.7 | 11 |
| Chilled seed | 51 | 700-950 | 30 $\mu g/105$ g[2] | 22 | 4.6 | 150 | 0.6 | 3.3 |
| Pericarp from dormant samara | 90 | ... | 30 $\mu g/100$ g[2] | 29 | 20 | 730 | 2.8 | 24 |
| Pericarp from chilled samara | 90 | ... | 40 $\mu g/90$ g[5] | 21 | 11 | 490 | 1.8 | 15 |
| *F. ornus* | | | | | | | | |
| Seed | 89 | 750 | 50 $\mu g/200$ g[2] | 22 | 6.3 | 110 | 0.4 | 2.6 |
| Pericarp | 90 | ... | 45 $\mu g/100$ g[2] | 22 | 6.8 | 220 | 0.8 | 1.6 |

[1] Calculated on dry weight basis.
[2] Fresh weight of sample.
[3] Calculated from UV spectral data.
[4] Calculated from ORD data.
[5] Weight after drying in vacuum over phosphorus pentoxide at room temperature.

$GA_3$ germination takes place a day earlier and therefore leads to an increase in $\Sigma 10$ to about 850.

The relative sizes of pericarp, seed, excised embryo before and after germination is seen in figure 1. The pericarp accounts for 55 and 26 % of the total weight of the *F. americana* and *F. ornus* samara respectively. The weight loss during cold-temperature after-ripening of *F. americana* is 5 % in the pericarp and 10 % in the depericarped seed.

The data in table I show that (S)-ABA is present in the samaras of both *Fraxinus* species, is highest in the dormant *F. americana* samaras, and decreases during cold-temperature after-ripening. The most striking effect of the cold treatment is the decrease of the ABA level in the seed of *F. americana.* On a dry weight basis this brings the ABA level of cold-treated *F. americana* seeds into the same concentration range as that found in nondormant *F. ornus* seeds. The decrease in the ABA level of the *F. americana* pericarp during cold-temperature after-ripening is less pronounced. It should also be noted that on a dry weight basis the pericarp of both species contain more ABA than the seed irrespective of the state of dormancy.

In the last column of table I the ABA concentrations are expressed as ABA per 1000 dry pericarps or 1000 dry seeds. On this basis, *F. americana* samaras contain 31 % of their ABA in the seed before cold treatment but only 18 % afterwards. When the ABA concentrations of seeds are compared it is seen that dormant seeds from *F. americana* contain the largest amount. However, after cold-temperature after-ripening the ABA level of seeds from *F. americana* is approximately as low as that found in the seeds of *F. ornus.*

Before the physiological relevance of these data can be evaluated 2 questions must be answered. Does the ABA in the pericarp play a role in seed germination and is the decrease in ABA during cold-temperature after-ripening of physiological significance? Our work to date leads us to conclude that the ABA found in the pericarp of these 2 species is probably of little significance to seed germination, while the decrease in the ABA level of the seed during cold-treatment may have physiological importance to germination.

If the ABA in the pericarp is to contribute to seed dormancy it must diffuse through the seed coat into the seed. However, we have been unable to delay germination by substituting ABA solutions for pericarps. In these experiments a special germination procedure had to be used. This became

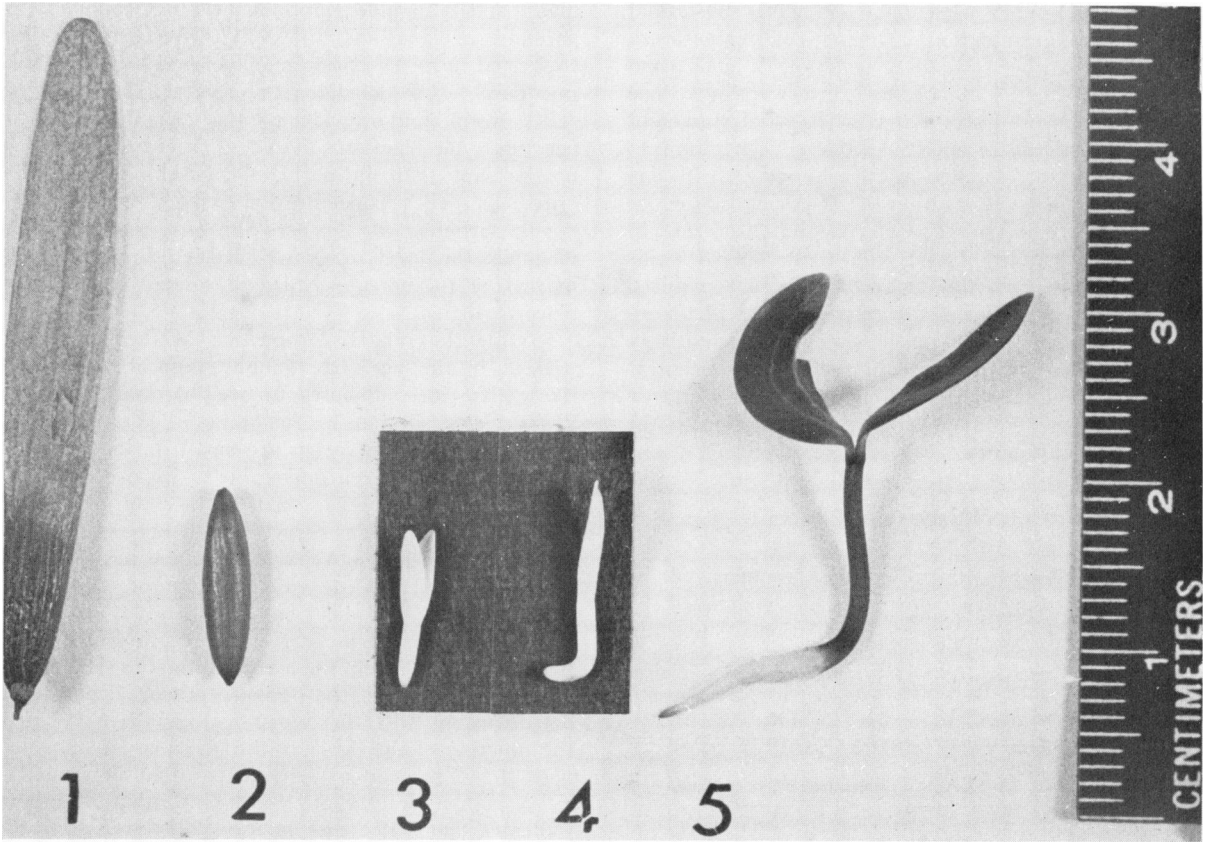


FIG. 1. Relative sizes of *Fraxinus americana* L. pericarp, seed and embryo before and after germination. 1) Intact samara (seed enclosed in pericarp). 2) Seed after 24 hour imbibition. 3) Embryo immediately after excision from seed that had been imbibed 24 hours. 4) Germinated embryo, 24 to 48 hours after excision. 5) Ten day old seedling from excised embryo.

necessary when it was observed that excised nondormant embryos will germinate on wet filter paper unless they are continuously exposed to an ABA solution of the proper concentration. If the excised embryos are placed on dry microscope slides in a petri dish which has wet filter paper below the microscope slide it is no longer necessary to expose them continuously to ABA in order to prevent germination. While the embryos do not develop as fully under these conditions as when placed directly on wet filter paper, curvature is interfered with least. Using this procedure we found complete suppression of germination when excised nondormant embryos were soaked for 24 hours in 10 $\mu$M (RS)-ABA and then placed into the moist chambers. In the absence of exogenously added (RS)-ABA $\Sigma 10 = 450$ under these conditions. If, however, the intact seeds were soaked in (RS)-ABA solutions, and excised embryos from the seeds placed on microscope slides in moist chambers, no decrease in $\Sigma 10$ was observed. This was true even if the seeds were soaked in 100 $\mu$M (RS)-ABA for 2 weeks at 5°.

In order to determine the possible significance of the decrease in (S)-ABA during cold-temperature after-ripening, we determined the effects of (RS)-ABA on the germination of embryos excised from cold-treated seeds, table II. The lowest concentration of (RS)-ABA used, 0.95 $\mu$M, gave substantial inhibition of germination. During cold-temperature after-ripening the (S)-ABA level of *F. americana* seeds dropped 1.1 $\mu$mole/kg dry weight. Since excised embryos have about a 50% dry weight content the decrease in (S)-ABA concentration in embryos is 0.55 $\mu$mole/kg fresh weight if one assumes uniform distribution of ABA between endosperm and embryo. Therefore, the decrease in (S)-ABA observed during cold-treatment of seeds is of the same order of magnitude as that required for the partial inhibition of germination with exogenously applied ABA. It may thus be tentatively concluded that the decrease in the ABA concentration in *F. americana* seeds during cold-temperature after-ripening is of physiological significance to the breaking of dormancy.

## Discussion

There is substantial evidence, summarized by Amen (2), that seed dormancy is controlled by hormonal inhibitor-promoter regulation. This applies not only to such seeds as ash in which true embryo dormancy is found but also to others, such as lettuce, in which regulation of radicle protrusion is the only dormancy control. Just how significant a role abscisic acid has in this regulation is still not clear. From our data it would appear that in *F. americana* seeds ABA does contribute to seed dormancy. High levels were found in the seed before cold-temperature after-ripening. During the chilling treatment the level in the seed dropped to the same range as that found in nondormant *F. ornus*. This decrease is believed to have physiological significance to dormancy in *F. americana* since (RS)-ABA at a concentration of 0.95 $\mu$M can reimpose a substantial degree of dormancy. On the basis of the assumptions outlined in the previous section, this is in the same concentration range as the ABA lost during the chilling treatment. Nevertheless, it is not claimed that the addition of ABA to nondormant embryos reimposes true embryo dormancy. Inhibition of germination of a nondormant *Fraxinus* embryo with (RS)-ABA requires continuous exposure to this substance. This is not true of embryos from dormant *F. americana* where little if any germination occurs during a 10 day period. This would indicate that the decrease in (S)-ABA concentration is not the only difference between a dormant and a nondormant embryo. A number of possible explanations for this difference can be suggested: 1) Differences in permeability or binding may be responsible for greater "leakage" of ABA from nondormant embryos. 2) The rate of enzymatic destruction of ABA may be higher in nondormant than in dormant embryos. 3) A decrease in ABA concentration while required may not be sufficient for the breaking of dormancy. For example, the level of growth-accelerating hormones may have to increase before germination can take place.

Table II. *Effects of (RS)-Abscisic Acid on Germination, Growth, and Chlorophyll Content of Nondormant* Fraxinus americana *Embryos*

| (RS)-ABA | Germination as $\Sigma 10$ | Fr wt increase | Total chlorophyll content |
|---|---|---|---|
| *μmole/l* | | % | *mg/100 g fr wt* |
| 0 | 960 | 279 | 34 |
| 0.95 | 540 | 101 | 13 |
| 3.80 | 210 | 23 | 0 |

While the relative proportion of the (S)-ABA present in the pericarp is quite high it seems unlikely that it contributes to seed dormancy in the 2 *Fraxinus* species we examined. The losses of (S)-ABA in the pericarp are small during the cold treatment; it has been impossible to impose germination inhibition on whole seeds when they are bathed in high concentrations of ABA and there is no clear relation between the ABA level in the pericarp and the physiological state of the embryo. Since the percentage of ABA lost was significantly greater in the seed than in the pericarp it is tempting to suggest that the loss inside the seed is due to enzymatic action while that in the pericarp is caused primarily by diffusion into the moist vermiculite. Possibly the function of the ABA in the pericarp is connected with the regulation of senescence or abscission.

A comparison of our work with that of others indicates that a decrease in the ABA levels of seeds during cold temperature after-ripening is not a general control mechanism for the breaking of dormancy of seeds with a chilling requirement. Pos-

sibly the dormancy regulation in peach seeds comes closest to that encountered in *F. americana*. Lipe and Crane (5) concluded from chromatographic and UV spectral data that the germination inhibitor in peach seeds is ABA and interpret their bioassay as indicating a decrease in the inhibitor concentration during chilling. Villiers and Wareing, on the other hand, found that in *F. excelsior* the concentration of inhibitor does not change during chilling but that the level of accelerator increases (11). This report was published before ABA had been discovered but judging from the chromatographic characteristics of their inhibitor further work may show the material to be ABA. The *F. excelsior* seeds differ from *F. americana* in that the seed contains an immature embryo which requires after-ripening near 25° prior to chilling, and leaching of the excised embryo for 48 hours at room temperature can replace the chilling requirement. An examination of dormant hazel seeds (*Corylus avellana* L.) by Bardbeer (3) showed that while the dormant seeds contain germination inhibitors, ABA if at all present, accounts for less than 1% of the total inhibitory activity. Since Amo 1618 prevented or delayed the onset of germination it was suggested that the breaking of dormancy during the cold-temperature after-ripening may be attributed to the synthesis of gibberellin.

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